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Bacillus thuringiensis strains and their genes encoding insecticidal toxins

Abstract

Two new Bacillus thuringiensis strains, which are deposited at the DSM under accession numbers 5870 and 5871, produce new crystal proteins during sporulation that are toxic to Coleoptera and that are encoded by new genes. The crystal proteins contain protoxins, which can yield toxins as trypsin-digestion products. A plant, the genome of which is transformed with a DNA sequence that comes from either one of the strains and encodes an insecticidally effective portion of its respective protoxin or encodes its respective toxin, is resistant to Coleoptera. Each strain, itself, or its crystals, crystal proteins, protoxin, toxin and/or insecticidally effective protoxin portion can be used as the active ingredient in an insecticidal composition for combatting Coleoptera.

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Parent Case Text

This application is a divisional of application Ser. No. 07/952,755, filed Nov. 17, 1992 now U.S. Pat. No. 5,466,597.

Claims

1. A transformed plant cell comprising a chimeric gene comprising an isolated DNA sequence encoding a Btl109P protein of SEQ. ID. No. 1; or an insecticidally effective part of the Btl109P protein of SEQ. ID. No. 1, or a truncated Btl109P protein of SEQ. ID. No. 1 having at least the toxin activity of the Btl109P protein, said DNA being under the control of a plant expressible promoter.

2. A plant or a seed thereof comprising the plant cell of claim 1.

3. A plant genome including, integrated therein, an isolated btl109P gene encoding a btl109P protein comprising the amino acid sequence of SEQ. ID. No. 1 or an insecticidally effective part of the Btl109P protein of SEQ. ID. No. 1 or a truncated Btl109P protein of SEQ. ID. No. 1 having at least the toxin activity of the Btl109P protein.

4. A plant tissue, the cells of which have the plant genome of claim 3.

5. A process for rendering a plant resistant to Leptinotarsa decemlineata comprising transforming a plant genome with an isolated btl109P gene encoding a btl109P protein comprising the amino acid sequence of SEQ ID No. 1 or an insecticidally effective part of the Btl109P protein of SEQ ID No. 1 or a truncated Btl109P protein of SEQ ID No. 1 having at least the toxin activity of the Btl109P protein of SEQ ID No. 1.

6. A process for transforming plants which comprises the steps of transforming plant cells with a DNA sequence encoding the protein of SEQ ID No. 1 or an insecticidally effective part thereof and regenerating said transformed plant cells into plants and reproduction material thereof comprising a DNA sequence encoding said protein or said insecticidally effective part.

Description

BACKGROUND OF THE INVENTION

(i) Field of the Invention

This invention relates to two new strains of B. thuringiensis (the "Btl109P strain" and the "Btl260 strain"), each of which produces crystallized proteins (the "Btl109P crystal proteins" and the "Btl260 crystal proteins", respectively) which are packaged in crystals (the "Btl109P crystals" and the "Btl260 crystals", respectively) during sporulation. The Btl109P and Btl260 strains were deposited under the provisions of the Budapest Treaty at the Deutsche Sammlung Fur Mikroorganismen and Zellkulturen ("DSM"), Mascheroder Weg 1B, D-3300 Braunschweig, Federal Republic of Germany, under accession numbers 5870 and 5871, respectively, on Apr. 4, 1990.

This invention also relates to an insecticide composition that is active against Coleoptera and that comprises the Btl109P or Btl260 strain, as such, or preferably the Btl109P or Btl260

crystals, crystal proteins or the active component(s) thereof as an active ingredient.

This invention further relates

1) The "btl109P gene", from the genome of the Btl109P strain, which encodes an insecticidal protein (the "Btl109P protoxin") that is found in the Btl109P crystals; and

2) The "btl260 gene", from the genome of the Btl260 strain, which encodes an insecticidal protein (the "Btl260 protoxin") that is found in the Btl260 crystals.

The Btl109P and Btl260 protoxins are the proteins that are produced by their respective Btl109P and Btl260 strains before being packaged into their respective Btl109P and Btl260 crystals.

This invention still further relates to the "Btl109P toxin" and the "Btl260 toxin" which can be obtained (e.g., by trypsin digestion) from the Btl109P protoxin and the Btl260 protoxin, respectively. The Btl109P and Btl260 toxins are insecticidally active proteins which can be liberated from the Btl109P crystals and the Btl260 crystals, respectively, produced by the Btl109P strain and the Btl260 strain, respectively. Each toxin has a high activity against Coleoptera. The Btl109P and Btl260 toxins are believed to represent the smallest portions of their respective Btl109P and Btl260 protoxins which are insecticidally effective against Coleoptera.

This invention yet further relates to a chimaeric gene that can be used to transform a plant cell and that contains:

1) a part of the btl109P or btl260 gene (the "insecticidally effective btl109P or btl260 gene part") encoding an insectidicidally effective portion of the respective Btl109P or Btl260 protoxin, preferably a truncated part of the btl109P or btl260 gene (the "truncated btl109P or btl260 gene") encoding just the respective Btl109P or Btl260 toxin;

2) a promoter suitable for transcription of the insecticidally effective btl109P or btl260 gene part in a plant cell; and

3) suitable 3' end transcript formation and polyadenylation signals for expressing the insecticidally effective btl109P or btl260 gene part in a plant cell.

This chimaeric gene is hereinafter generally referred to as the "btl109P or btl260 chimaeric gene." Preferably, the insecticidally effective btl109P or btl260 gene part is present in the btl109P or btl260 chimaeric gene as a hybrid gene comprising a fusion of the truncated btl109P or btl260 gene and a selectable marker gene, such as the neogene (the "btl109P-neo or btl260-neo hybrid gene") encoding a Btl109P-NPTII or Btl260-NPTII fusion protein.

This invention also relates to:

1) a cell (the "transformed plant cell") of a plant, such as potato or corn, the nuclear genome of which is transformed with the insecticidally effective btl109P or btl260 gene part; and

2) a plant (the "transformed plant") which is regenerated from the transformed plant cell or is produced from the so-regenerated plant, the nuclear genome of which contains the insecticidally effective btl109P or btl260 gene part and which is resistant to Coleoptera.

This invention still further relates to a B. thuringiensis ("Bt") strain transformed, preferably by electroporation, with a vector carrying all or part of the btl109P or btl260 gene.

(ii) Description of Related Art

B. thuringiensis ("Bt") is a gram-positive bacterium which produces endogenous crystals upon sporulation. The crystals are composed of proteins which are specifically toxic against insect larvae. Three different Bt pathotypes have been described: pathotype A that is active against Lepidoptera, e.g., caterpillars; pathotype B that is active against certain Diptera, e.g., mosquitos and black flies; and pathotype C that is active against Coleoptera, e.g., beetles (Ellar et al, 1986).

A Bt strain, whose crystals are toxic to Coleoptera, has been described as Bt tenebrionis (U.S. Pat. No. 4,766,203; European patent publication ("EP") 149,162), as Bt M-7 or Bt San Diego (EP 213,818; U.S. Pat. No. 4,771,131) and as BtS1 (European patent application ("EPA") 88402115.5). Two other strains toxic to Coleoptera, BtPGSI208 and BtPGSI245, have also been described (PCT publication WO 90/09445).

The fact that conventional submerged fermentation techniques can be used to produce Bt spores on a large scale makes Bt bacteria commercially attractive as a source of insecticidal compositions.

Gene fragments from some Bt strains, encoding insecticidal proteins, have heretofore been identified and integrated into plant genomes in order to render the plants insect-resistant. However, obtaining expression of such Bt gene fragments in plants is not a straightforward process. To achieve optimal expression of an insecticidal protein in plant cells, it has been found necessary to engineer each Bt gene fragment in a specific way so that it encodes a water-soluble part of a Bt protoxin that retains substantial toxicity against its target insects (EPA 86300291.1 and EPA 88402115.5; U.S. patent application Ser. No. 821,582, filed Jan. 22, 1986).

SUMMARY OF THE INVENTION

In accordance with this invention, the two new Bt strains of pathotype C, i.e., the Btl109P and Btl260 strains, are provided. The Btl109P and Btl260 crystals, crystal proteins, protoxins and toxins, produced by the respective strains during sporulation, as well as insecticidally effective portions of the Btl109P and Btl260 protoxins, each possess insecticidal activity and can therefore be formulated into insecticidal compositions against Coleoptera in general, especially

against Agelastica alni, Diabrotica luteold, Haltica tombacina, Anthonomus grandis, Tenebrio molitor, Diabrotica undecimpunctata, Triboleum castaneum, Dicladispa armigera, Trichispa serica, Oulema oryzae, Colaspis brunnea, Lissorhorptrus oryzophilus, Phyllotreta cruciferae, Phyllotreta strtolata, Psylliodes punctulata, Entomoscelis americana, Meligethes aeneus, Ceutorynchus sp., Psylliodes chrysocephala, and Phyllotreta undulata and particularly against the Colorado potato beetle, Leptinotarsa decemlineata, which is a major pest of economically important crops.

Also in accordance with this invention, a plant cell genome is transformed with the insecticidally effective btl109P or btl260 gene part, preferably the truncated btl109P or btl260 gene. It is preferred that this transformation be carried out with the btl109P or btl260 chimaeric gene. The resulting transformed plant cell can be used to produce a transformed plant in which the plant cells in some or all of the plant tissues: 1) contain the insecticidally effective btl109P or btl260 gene part as a stable insert in their genome and 2) express the insecticidally effective btl109P or btl260 gene part by producing an insecticidally effective portion of its respective Btl109P or Btl260 protoxin, preferably its respective Btl109P or Btl260 toxin, thereby rendering the plant resistant to Coleoptera. The transformed plant cells of this invention can also be used to produce, for recovery, such insecticidal Bt proteins.

Further in accordance with this invention, a process is provided for rendering a plant resistant to Coleoptera by transforming the plant cell genome with the insecticidally effective btl109P or btl260 gene part, preferably the truncated btl109P or btl260 gene. In this regard, it is preferred that the plant cell be transformed with the btl109P or btl260 chimaeric gene.

Still further in accordance with this invention, there are provided the Btl109P and Btl260 protoxins, the insecticidally effective portions of such protoxins and the Btl109P and Btl260 toxins, as well as the btl109P and btl260 genes, the insecticidally effective btl109P and btl260 gene parts, the truncated Btl109P and btl260 genes and the chimaeric btl109P and btl260 genes.

Yet further in accordance with this invention, a Bt strain is transformed, preferably by electropotation, with a vector carrying all or part of the btl109P or btl260 gene encoding all or an insecticidally effective portion of the Btl109P or Btl260 protoxin.

Also in accordance with this invention are provided an insecticidal composition against Coleoptera and a method for controlling Coleoptera with the insecticidal composition, wherein the insecticidal composition comprises the Btl260 or Btl109P strain, crystals, crystal proteins, protoxin, toxin and/or insecticidally effective protoxin portions.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the total protein patterns by SDS-PAGE of sporulated Btl109P, Btl260, BtS1 and BtPGSI208 Bacillus cultures;

FIG. 2 shows the hybridization patterns under low stringency conditions of EcoRI digested total

DNA prepared from strains BtS1, BtPGSI208, BtI109P and BtI260 with a PstI-EcoRV fragment of the genome of the BtS1 strain;

FIGS. 3A-B show the hybridization pattern under low stringency conditions of NlaIV digested total DNA prepared from strains BtS1, BtPGSI208, BtI109P and BtI260 with a 1.38 kb EcoRV-NcoI fragment of the genome of the BtPGSI208 strain.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In accordance with this invention, the BtI109P and BtI260 protoxins can be isolated in a conventional manner from, respectively, the BtI109P strain, deposited at the DSM under accession number 5870, and the BtI260 strain, deposited at the DSM under accession number 5871. For example, the BtI109P and BtI260 crystals can be isolated from sporulated cultures of their respective strains (Mahillon and Delcour, 1984), and then, the respective protoxins can be isolated from these crystals according to the method of Hofte et al (1986). The protoxins can be used to prepare monoclonal or polyclonal antibodies specific for these protoxins in a conventional manner (Hofte et al, 1988). The BtI109P toxin can then be obtained by protease digestion (e.g., by trypsin digestion) of the BtI109P protoxin. The BtI260 toxin can be obtained by protease digestion (e.g., by trypsin digestion) of the BtI260 protoxin.

The btI109P and btI260 genes can also be isolated from their respective strains in a conventional manner. For example, the btI109P or btI260 gene can be identified in its respective BtI109P or BtI260 strain, using the procedure described in U.S. patent application Ser. No. 821,582 and in EPA 86300291.1 and EPA 88402115.5 (which are incorporated herein by reference). Preferably, the btI109P and btI260 genes are each identified by: digesting total DNA from their respective BtI109P and BtI260 strains with one or more restriction enzymes; size fractionating the DNA fragments, So produced, into DNA fractions of 5 to 10 Kb; ligating such fractions to cloning vectors; transforming E. coli with the cloning vectors; and screening the clones with a suitable DNA probe. The DNA probe can be constructed: 1) from a highly conserved region of a bt gene which encodes another crystal protoxin against Coleoptera such as: the bt13 gene described in EPA 88402115.5 and by Hofte et al (1987); or 2) on the basis of the N-terminal amino acid sequence of the protoxin encoded by the respective btI109P or btI260 gene, which sequence can be determined by gas-phase sequencing of the immobilized protoxin (EPA 88402115.5).

Alternatively, the 5 to 10 kB fragments, prepared from total DNA of the BtI109P or BtI260 strain, can be ligated in suitable expression vectors and transformed in E. coli. The clones can then be screened by conventional colony immunoprobing methods (French et al, 1986) for expression of the BtI109P or BtI260 toxin with monoclonal or polyclonal antibodies raised against the toxin.

The so-identifed btI109P and btI260 genes can then each be sequenced in a conventional manner (Maxam and Gilbert, 1980) to obtain the DNA sequences. Hybridizations in Southern blots indicate that these genes are different from previously described genes encoding protoxins

and toxins with activity against Coleoptera (Hofte and Whiteley, 1989).

An insecticidally effective part of each of the genes, encoding an insecticidally effective portion of its protoxin, and a truncated part of each of the sequenced genes, encoding Just its toxin, can be made in a conventional manner from each gene after the gene has been sequenced. The amino acid sequences of the Btl109P and Btl260 protoxins and toxins can further be determined from the DNA sequences of their respective btl109P and btl260 genes and truncated btl109P and btl260 genes. By "an insecticidally effective part" or "a part" of the btl109P or btl260 gene is meant a DNA sequence encoding a polypeptide which has fewer amino acids then the respective Btl109P or Btl260 protoxin but which is still toxic to Coleoptera. Such a part of the btl109P or btl260 gene can encode a Btl109P or Btl260 protoxin which has been truncated towards at least one trypsin cleavage site of the protoxin (U.S. patent application Ser. No. 821,582; EPA 86300291.1).

In order to express all or an insecticidally effective part of the btl109P or btl260 gene in E. coli and in plants, suitable restriction sites can be introduced, flanking each gene or gene part. This can be done by site directed mutagenesis, using well-known procedures (Stanssens et al, 1987; Stanssens et al, 1989).

The insecticidally effective btl109P or btl260 gene part, encoding an insecticidally effective portion of its respective Btl109P or Btl260 protoxin, can be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell be used in a conventional manner to produce a transformed plant that is insect-resistant. In this regard, a disarmed Ti-plasmid, containing the insectidicidally effective btl109P or btl260 gene part, in Agrobacterium tumefaciens can be used to transform the plant cell, and thereafter, a transformed plant can be regenerated from the transformed plant cell using the procedures described, for example, in EP 116,718 and EP 270,822, PCT publication WO 84/02,913, EPA 87400544.0 and Gould et al. (1991) (which are also incorporated herein by reference). Preferred Ti-plasmid vectors each contain the insecticidally effective btl109P or btl260 gene part between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 233,247), pollen mediated transformation (as described, for example, in EP 270,356, PCT publication WO 85/01856, and U.S. Pat. No. 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 67,553 and U.S. Pat. No. 4,407,956), liposome-mediated transformation (as described, for example, U.S. Pat. No. 4,536,475), and other methods such as the recently described methods for transforming certain lines of corn (Fromm et al, 1990; Gordon-Kamm et al, 1990).

The resulting transformed plant can be used in a conventional plant breeding scheme to produce more transformed plants with the same characteristics or to introduce the insecticidally effective btl109P or btl260 gene part in other varieties of the same or related plant species. Seeds, which are obtained from the transfomed plants, contain the insecticidally effective btl109P or btl260 gene part as a stable genomic insert. Cells of the transformed plant can be cultured in a conventional manner to produce the insecticidally effective portion of the

respective Btl109P or Btl260 protoxin, preferably the respective Btl109P or Btl260 toxin, which can be recovered for use in conventional insecticide compositions against Coloptera (U.S. patent application Ser. No. 821,582; EPA 86300291.1.).

The insecticidally effective btl109P or btl260 gene part, preferably the truncated btl109P or btl260 gene, is inserted in a plant cell genome so that the inserted part of the gene is downstream (i.e., 3') of, and under the control of, a promoter which can direct the expression of the gene part in the plant cell. This is preferably accomplished by inserting the btl109P or btl260 chimaeric gene in the plant cell genome. Preferred promoters include: the strong constitutive 35S promoters (the "35S promoters") of the cauliflower mosaic virus of isolates CM 1841 (Gardner et al, 1981), CabbB-S (Franck et al, 1980) and CabbB-JI (Hull and Howell, 1987); and the TR1' promoter and the TR2' promoter (the "TR1' promoter" and "TR2' promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten et al, 1984). Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (e.g., leaves and/or roots) whereby the inserted btl109P or btl260 gene part is expressed only in cells of the specific tissue(s) or organ(s). For example, the btl109P or btl260 gene part could be selectively expressed in the leaves of a plant (e.g., potato, corn, oilseed rape and rice) by placing the gene part under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in U.S. patent application Ser. No. 821,582 and EPA 86300291.1. Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical factors).

The insecticidally effective btl109P or btl260 gene part is inserted in the plant genome so that the inserted part of the gene is upstream (i.e., 5') of suitable 3' end transcription regulation signals (i.e., transcript formation and polyadenylation signals). This is preferably accomplished by inserting the btl109P or btl260 chimaeric gene in the plant cell genome. Preferred polyadenylation and transcript formation signals include those of the octopine synthase gene (Gielen et al, 1984) and the T-DNA gene 7 (Velten and Schell, 1985), which act as 3'-untranslated DNA sequences in transformed plant cells.

It is preferred that the insecticidally effective btl109P or btl260 gene part be inserted in the plant genome in the same transcriptional unit as, and under the control of, the same promoter as a selectable marker gene. The resulting hybrid btl109P or btl260-marker gene will, thereby, be expressed in a transformed plant as a fusion protein (U.S. patent application Ser. No. 821,582; EPA 86300291.1; Vaeck et al, 1987). This result can be preferably accomplished by inserting a btl109P or btl260 chimaeric gene, containing the marker gene, in the plant cell genome. Any conventional marker gene can be utilized, the expression of which can be used to select transformed plant cells. An example of a suitable selectable marker gene is an antibiotic resistance gene such as the neo gene coding for kanamycin resistance (Reiss et al, 1984; EPA 87400544.0; U.S. patent application Ser. No. 821,582; EPA 86300291.1). Thereby, the insecticidally effective btl109P or btl260 gene part and the marker gene (e.g., the btl109P-neo or btl260-neo hybrid gene) are expressed in a transformed plant as a fusion protein (U.S. patent application Ser. No. 821,582; EPA 86300291.1; Vaeck et al, 1987).

All or an insecticidally effective part of the btl109P and btl260 genes, encoding Coleopteran toxins, can also be used to transform gram-positive bacteria, such as a B. thuringiensis which has insecticidal activity against Lepidoptera or Coleoptera. Thereby, a transformed Bt strain can be produced which is useful for combatting both Lepidopteran and Coleopteran insect pests or combatting additional Coleopteran insect pests. Transformation of a bacteria with all or part of the btl109P or btl260 gene, incorporated in a suitable cloning vehicle, can be carried out in a conventional manner, preferably using conventional electroporation techniques as described in PCT patent application PCT/EP89/01539, filed Dec. 11, 1989.

The Btl109P or Btl260 strain also can be transformed with all or an insecticidally effective part of one or more foreign Bt genes such as: the bt2 gene (U.S. patent application Ser. No. 821,582; EPA 86300291.1) or another Bt gene coding for all or an insecticidally effective portion of a Bt protoxin active against Lepidoptera; and/or the bt13 gene (EPA 88402115.5) or another Bt gene, such as the btPGSI208 gene or btPGSI245 gene (EPA 89400428.2; PCT publication WO 90/09445), coding for all or an insecticidally effective portion of a Bt protoxin active against Coleoptera. Thereby, a transformed Bt strain can be produced which is useful for combatting an even greater variety of insect pests, e.g., Lepidoptera and/or additional Coleoptera. Transformation of the Btl109P or Btl260 strain with all or part of a foreign Bt gene, incorporated in a conventional cloning vector, can be carried out in a well known manner, preferably using conventional electroporation techniques (Chassy et al, 1988).

Each of the Btl109P and Btl260 strains can be fermented by conventional methods (Dulmage, 1981) to provide high yields of cells. Under appropriate conditions which are well understood (Dulmage, 1981), the Btl109P and Btl260 strains each sporulate to provide their respective Btl109P and Btl260 crystal proteins in high yields.

The Btl109P and Btl260 strains, crystals, protoxins, toxins and/or insecticidally effective portions, preferably their protoxins, can each be used as the active ingredient in an insecticide composition used to control insect pests belonging to the order of Coleoptera. For example, the Btl109P or Btl260 crystals can be isolated from sporulated cultures of the Btl109P or Btl260 strain (Mahillon and Delcour, 1984), and then, the respective protoxin can be isolated from these crystals according to the method of Hofte et al (1986).

An insecticidal, particularly anti-Coleopteran, composition of this invention can be formulated in a conventional manner using the Btl109P or Btl260 strain or preferably its respective crystals, crystal proteins, protoxin, toxin and/or insecticidally effective portion of its protoxin as active ingredient(s), together with suitable carriers, diluents, emulsifiers and/or dispersants. This insecticide composition can be formulated as a wettable powder, pellets, granules or dust or as a liquid formulation with aqueous or non-aqueous solvents as a foam, gel, suspension, concentrate, etc. The concentration of the Btl109P or Btl260 strain, crystals, crystal proteins, protoxin, toxin and/or insecticidally effective protoxin portion in such a composition will depend upon the nature of the formulation and its intended mode of use. Generally, an insecticide composition of this invention can be used to protect a potato field for 2 to 4 weeks

against Coleoptera with each application of the composition. For more extended protection (e.g., for a whole growing season), additional amounts of the composition should be applied periodically.

A method for controlling insects, particularly Coleoptera, in accordance with this invention preferably comprises applying (e.g., spraying), to a locus (area) to be protected, an insecticidal amount of the Btl109P or Btl260 crystals, crystal proteins, protoxin, toxin or insecticidally effective protoxin portion, preferably protoxin. The locus to be protected can include, for example, the habitat of the insect pests or growing vegetation or an area where vegetation is to be grown.

To obtain the Btl109P or Btl260 protoxin or toxin, cells of the Btl109P or Btl260 strain can be grown in a conventional manner on a suitable culture medium and then lysed using conventional means such as enzymatic degradation or detergents or the like. The protoxin can then be separated and purified by standard techniques such as chromatography, extraction, electrophoresis, or the like. The toxin can then be obtained by trypsin digestion of the protoxin.

The Btl109p or Btl260 cells also can be harvested and then applied intact, either alive or dead, preferably dried, to the locus to be protected. In this regard, it is preferred that a purified Btl109P or Btl260 strain (either alive or dead) be used, particularly a cell mass that is 90.0 to 99.9% Btl109P or Btl260 strain.

The Btl109P or Btl260 cells, crystals, crystal proteins, protoxin, toxin, or insecticidally effective protoxin portion can be formulated in an insecticidal composition in a variety of ways, using any number of conventional additives, wet or dry, depending upon the particular use. Additives can include wetting agents, detergents, stabilizers, adhering agents, spreading agents and extenders. Examples of such a composition include pastes, dusting powders, wettable powders, granules, baits and aerosol compositions. Other Bt cells, crystals, crystal proteins, protoxins, toxins, and insecticidally effective protoxin portions and other insecticides, as well as fungicides, biocides, herbicides and fertilizers, can be employed along with the Btl109P or Btl260 cells, crystals, crystal proteins, protoxin, toxin and/or insecticidally effective protoxin portion to provide additional advantages or benefits. Such an insecticidal composition can be prepared in a conventional manner, and the amount of the Btl109P or Btl260 cells, crystals, crystal proteins, protoxin, toxin, and/or insecticidally effective protoxin portion employed depends upon a variety of factors, such as the insect pest targeted, the composition used, the type of area to which the composition is to be applied, and the prevailing weather conditions. Generally, the concentration of the Btl109P or Btl260 protoxin, insecticidally effective protoxin portion and/or toxin will be at least about 0.1% of the weight of the formulation to about 100% by weight of the formulation, more often from about 0.15% to about 0.8% weight percent of the formulation.

In practice, some insects can be fed the Btl109P or Btl260 protoxin, toxin, insecticidally effective protoxin portion or mixtures thereof in the protected area, that is, in the area where such protoxin, toxin and/or insecticidally effective protoxin portion have been applied.

Alternatively, some insects can be fed intact and alive cells of the Btl109P or Btl260 strain or transformants thereof, so that the insects ingest some of the strain's protoxin and suffer death or damage.

The following Examples illustrate the invention. The Figures and Sequence Listing, referred to in the Examples, are as follows:

Figures

FIG. 1--Total protein patterns by SDS-PAGE of sporulated Btl109P, Btl260, BtS1 and BtPGSI208 Bacillus cultures. "MW" designates molecular weight markers.

FIG. 2--Hybridisation pattern under low stringency conditions of EcoRI digested total DNA prepared from strains BtS1, BtPGSI208, Btl109P and Btl260 with a 1.46 kb PstI-EcoRV fragment of the genome of the BtS1 strain, containing an internal fragment of the bt13 gene ("cryIIIA" gene) as probe.

FIG. 3--Hybridisation pattern under low stringency conditions of NlaIV digested total DNA prepared from strains BtS1, BtPGSI208, Btl109P and Btl260 with a 1.38 kb EcoRV-NcoI fragment of the genome of the BtPGSI208 strain, containing an internal fragment of the btPGSI208 gene ("cryIIIB" gene), as probe. Probe fragments were labeled with .sup.32 P (A) or with digoxygenin (B) (Boehringer Non-Radioactive Labeling Kit).

Sequence Listing

Seq. Id. No.1--DNA sequence of the btl109P gene. The derived aminoacid sequence of the encoded Btl109P protoxin is presented beneath the DNA sequence. The truncated btl109P gene, coding just for the Btl109P toxin, appears to extend from nucleotide position 397 to the TAA termination codon at nucleotide position 2179.

Seq. Id. No.2--Partial DNA sequence of the btl260 gene. The derived partial aminoacid sequence of the encoded Btl260 protoxin is presented beneath the DNA sequence.

Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA are carried out by the standardized procedures described in Maniatis et al, Molecular Cloning--A laboratory Manual, Cold Spring Harbor Laboratory (1982).

EXAMPLE 1

Characterization of the Btl109P and Btl260 strains.

The Btl109P strain was isolated from grain dust sampled in the Philippines and was deposited at the DSM on Apr. 4, 1990 under accession No. 5870.

The Btl260 strain was isolated from bat dung sampled in the Philippines and was deposited at

the DSM on Apr. 4, 1990 under accession No. 5871.

Each strain can be cultivated on conventional standard media, preferably LB medium (Bacto-tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l and agar 15 g/l), preferably at 28.degree. C. For long term storage, it is preferred to mix an equal volume of a spore-crystal suspension with an equal volume of 50% glycerol and store this at -70.degree. C or lyophilize a spore suspension. For sporulation, the use of T.sub.3 medium (tryprone 3 g/l, tryptose 2 g/l, yeast extract 1.5 g/l, 5 mg MnCl.sub.2, 0.05M Na.sub.2 PO.sub.4, pH 6.8 and 1.5% agar) is preferred for 24 hours at 28.degree. C., followed by storage at 4.degree. C. During its vegetative phase, each of the Btl109P and Btl260 strains can also grow under facultative anaerobic conditions, but sporulation only occurs under aerobic conditions.

Sterilization of each strain occurs by autoclave treatment at 120.degree. C. (1 bar pressure) for 20 minutes. Such treatment totally inactivates the spores and the crystalline Btl109P and Btl260 protoxins. UV radiation (254 nm) inactivates the spores but not the protoxins.

After cultivating on Nutrient Agar ("NA", Difco Laboratories, Detroit, Mich., USA) for one day, colonies of each of the Btl109P and Btl260 strains form opaque white colonies with irregular edges. Cells of each strain (Gram positive rods of 1.7-2.4.times.5.6-7.7 230 m) sporulate after three days cultivation at 28.degree. C. on NA. The crystal proteins produced during sporulation are packed in flat square crystals in the Btl109P strain and in small rhomboid crystals in the Btl260 strain. Both strains were further characterized by serotyping with B. thuringiensis H antisera (by B. de Barjac of Institut Pasteur, France). Btl109P belongs to serotype H 303b, at an agglutination titre of 25,000 with Bt kurstaki. Btl260 belongs to serotype H18, at an agglutination titre of 3,200 with Bt kumamotoensis.

EXAMPLE 2

Characteristics of the Btl109P and Btl260 crystals

The Btl109P and Btl260 strains were grown for 48 to 72 hours at 28.degree. C. on T.sub.3 medium. After sporulation, the spores and crystals were harvested in phosphate buffered saline solution ("PBS" from Oxoid Ltd., Basingstroke, Hampshire, U.K.). The resulting aqueous spore-crystal suspensions were centrifuged, and the pellets were resuspended in PBS, recentrifuged and the pellet resuspended again.

The total protein patterns of the sporulated cultures of Btl109P and Btl260 strains were compared (FIG. 1) to other Bacillus strains, which produce the CryIIIA or CryIIIB crystal proteins, according to Lambert et al (1987). For this comparison, an aliquot of the washed spore-crystal mixture of each strain was centrifuged, the supernatant discarded and the pellet solubilized in Sample Buffer Mix. The extracts containing crystal proteins, were analyzed on a 12.5% SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie brilliant blue R-250. The results of this analysis revealed the presence of a major band (molecular weight 65.5 kDa) and two minor bands (MW. 72.4 kDa and 49.1 kDa) in spore-crystals of strain Btl109P and two major bands of about 65 kDa and a band of about 30 kDa in spore-crystals of strain Btl260.

Furthermore, the overall protein patterns of BtI109P and BtI260 are clearly different from the overall protein pattern of BtS1.

EXAMPLE 3

Insecticidal activity of the BtI109P and BtI260 crystal proteins

As in Example 2, both strains were grown for 48 to 72 hrs at 28.degree. C. on T.sub.3 medium. After sporulation, the spores and crystals were harvested in PBS (phosphate buffered saline). The resulting spore-crystal suspensions were centrifuged, and the pellets were resuspended, recentrifuged and the pellets again resuspended after removal of the supernatant. The pellets were incubated overnight in aqueous solutions containing 50 mM Na.sub.2 CO.sub.3 and 5 mM dithiotreitol. After centrifugation, the supernatants were recovered, and the protein contents of the extracts of the respective crystal proteins of the two strains were determined.

Potato leaves were dipped either in standardized spore-crystal mixtures or in aqueous dilutions of the crystal protein solutions and then air dried for two hours. Colorado potato beetle larvae of the first instar were placed on the treated leaves, and mortality of the larvae was determined after three days. These results were compared with the mortality of larvae fed leaves treated with either spore-crystal mixtures or solubilized crystal proteins of BtS1 (from DSM, accession no. 4288) which was used as a reference strain. LC50 (50% lethal concentration), expressed either as ug of solubilized crystal proteins/ml solution or as the number of spore-crystals in the dip-suspension, was calculated by Probit analysis (Finney, 1971). The results, including the 95% confidence interval and the slope of the probit line, are summarized in Tables 1 and 2, below.

TABLE I

Comparison of the toxicity of solubilized crystal proteins from the BtI109P strain, the BtI260 strain, the Bt San Diego strain (NRRL accession no. B-15939) and the BtS1 strain (reference strain) against larvae of Leptinotarsa decemlineata.

Strain	LC50 ug/cm.sup.2	FL95min	FL95max	Slope
BtI109P	0.71	0.52	0.97	3.49
BtI260	6.76	4.71	9.71	2.10
BtS1	3.56	2.01	6.32	1.10
Bt SAN DIEGO	0.90	0.8	1.5	1.0

TABLE 2

Comparison of the toxicity of spore-crystal mixtures from the BtI109P strain, the BtI260 strain and the BtS1 strain (reference strain) against larvae of Leptinotarsa decemlineata.

	LC50 10.sup.a spore

Strain	crystals/ml	FL95min	FL95max	Slope
BtI109P	5.78	4.06	8.24	3.07
BtS1	3.24	2.37	4.42	4.18
BtI260	68.6	48.6	99.9	3.2
BtS1	8.5	6.2	11.4	4.9

EXAMPLE 4

Identification of the btl109P and btl260 genes

The Btl109P and Btl260 protoxins from the Btl109P and Btl260 strains respectively were detected by ELISA (Engvall and Pesce, 1978) with a polyclonal antiserum against the Bt13 coleopteran toxin (Hofts et al, 1987). The btl109P and btl260 genes were identified in their respective strains by preparing total DNA of these strains and then digesting the DNA with the restriction enzymes NlaIV and EcoRI.

The EcoRI-digested DNA was analyzed by Southern blotting, probing with a .sup.32 P labeled 1.46 kb. PstI-EcoRV fragment from the genome of the BtS1 strain (EPA 88402115.5) containing the bt13 gene. After hybridization with the probe, the blot was washed under low stringency conditions (2.times.SSC, 0.1% SDS at 68.degree. C. for 2.times.15 min) and developed. The autoradiogram (FIG. 2) shows that only the btl109P gene is related to the bt13 gene. The hybridization pattern with the probe also showed that the btl109P gene was clearly different from the bt13 gene and that the genome of the Btl260 strain did not contain DNA sequences that are related to the PstI-EcoRV probe fragment of bt13 (cryIIIA) under the experimental conditions used. (FIG. 2)

The NlaIV-digested DNA was analyzed by Southern blotting, probing with .sup.32 P-labeled or digoxygenin (Non-Radioactive Labeling Kit, Boehringer Mannheim, Mannheim, Germany) 1.38 kb EcoRV-NcoI fragment from the genome of the BtPGSI208 strain (PCT patent application PCT/EP90/00244) containing the btPGSI208 or cryIIIB gene. After hybridization with the probe, the blot was washed under low stringency conditions (2.times.SSC, 0.1% SDS at 68.degree. C. for 2.times.15 min) and developed. The results (FIG. 3) show that only the btl260 gene is related to the btPGSI208 gene. The hybridization pattern with the probe also showed that the btl260 gene was clearly different from the btPGSI208 gene and that the btl109P gene strain contains DNA sequences that are only distantly related to the btPGSI208 gene under the experimental conditions used (FIG. 3).

EXAMPLE 5

Cloning and expression of the btl109P gene

In order to isolate the btl109P gene, total DNA from the Btl109P strain was prepared.

Subsequently, total DNA was digested with HindIII. The digested DNA was size fractionated on a sucrose gradient, and fragments ranging from 5 kb to 7 kb were ligated to the HindIII-digested and BAP-treated cloning vector pUC19 (Yanisch-Perron et al, 1985). Recombinant E. coli clones, "pUC.cryIIIDHd1", containing the vector were then screened with an internal 1.4 kb EcoRV-PstI DNA fragment of the bt13 gene (EP 305,275), as a probe, to identify clones containing the btI109P gene.

The so-identified DNA fragments were then sequenced (Seq. Id. No. 1) according to Maxam and Gilbert (1980).

Based on the analysis of its DNA sequence, the gene is cut with appropriate restriction enzymes to give the truncated btI109P gene, encoding the BtI109P toxin.

EXAMPLE 6

Cloning and expression of the btI260 gene

In order to isolate the btI260 gene, total DNA from the BtI260 strain is prepared and partially digested with Sau 3A. The digested DNA is size fractioned on a sucrose gradient and fragments ranging from 5 Kb to 10 Kb are ligated to the BglII-digested and BAP-treated cloning vector pECOR251 (deposited under accession no. 4711 at DSM). Recombinant E.coli clones are then screened with an internal NcoI-EcoRV DNA fragment of the btPGSI208 gene (EP 382,990), as a probe, to identify clones containing the btI260 gene.

DNA fragments containing the btI260 gene are then sequenced (Seq. Id. no. 2) according to Maxam and Gilbert (1980).

Based on the analysis of its DNA sequence, the gene is cut with appropriate restriction enzymes to give the truncated btI260 gene encoding the BtI260 toxin.

EXAMPLE 7

Construction of a btI109P-neo hybrid gene and a btI260-neo hybrid gene

Following the procedure of U.S. patent application Ser. No. 821,582 and EPA 88402115.5 and EPA 86300291.1, the truncated btI109P and btI260 genes from Examples 5 and 6 are each fused to the neo gene to form the corresponding hybrid gene.

EXAMPLE 8

Insertion of the btI109P and btI260 genes, the truncated btI109P and btI260 genes and the btI109P-neo and btI260-neo hybrid genes in E. coli and insertion of the truncated btI109P and btI260 genes and the btI109P-neo and btI260-neo hybrid genes in potato plants

In order to express the btI109P gene and btI260 gene, the truncated btI109P gene and

truncated btl260 gene, and the btl109P-neo hybrid gene and btl260-neo hybrid gene from Examples 5, 6 and 7 in E. coli and in plants, different gene cassettes are made in E. coli according to the procedures described in EPA 86300291.1 and EPA 88402115.5.

To allow major expression in plants, cassettes, each containing one of the truncated and/or hybrid genes, are each inserted in an intermediate plant expression vector (between the T-DNA border sequences of this vector), are each fused to transcript formation and polyadenylation signals in the plant expression vector, are each placed under the control of a constitutive promoter such as the promoter from cauliflower mosaic virus driving the 35S3 transcript (Hull and Howell, 1987) or the 2' promoter from the TR-DNA of the octopine Ti-plasmid (Velten et al, 1984), and are each fused to 3' end transcript formation and polyadenylation signals capable of acting in plants, such as the 3' end of the octopine synthase gene (Gielen et al, 1984).

Using standard procedures (Deblaere et al, 1985), the intermediate plant expression vectors, containing the truncated btl109P and btl260 genes and the btl109P-neo and btl260-neo hybrid genes, are transferred into the Agrobacterium strain C 58 Cl Rif.sup.R (U.S. patent application Ser. No. 821,582; EPA 86300291.1) carrying the disarmed Ti-plasmid pGV2260 (Vaeck et al, 1987). Selection for spectinomycin resistance yields cointegrated plasmids, consisting of pGV2260 and the respective intermediate plant expression vectors. Each of these recombinant Agrobacterium strains is then used to transform different potato plants (Solanum tuberosum) so that the truncated btl109P gene, the truncated btl260 gene, the btl109P-neo hybrid gene and the btl260-neo hybrid gene are contained in, and expressed by, different potato plant cells.

EXAMPLE 9

Expression of the truncated btl109P and btl260 genes and the btl109P-neo and btl260-neo hybrid genes in potato plants

The insecticidal activity against Coleoptera of the expression products of the truncated btl109P and btl260 genes and the btl109P-neo and btl260-neo hybrid genes in leaves of transformed potato plants, generated from the transformed potato plant cells of Example 8, is evaluated by recording the growth rate and mortality of Leptinotarsa decemlineata larvae fed on these leaves. These results are compared with the growth rate of larvae fed leaves from untransformed potato plants. Toxicity assays are performed as described in EPA 88402115.5, U.S. patent application Ser. No. 821,582 and EPA 86300291.1. A significantly higher mortality rate is obtained among larvae fed on leaves of transformed potato plants containing the truncated btl109P gene, the truncated btl260 gene, the btl109P-neo hybrid gene or the btl260-neo hybrid gene than among larvae fed the leaves of untransformed plants.

Needless to say, this invention is not limited to the Btl109P (DSM 5870) strain and the Btl260 (DSM 5871) strain. Rather, the invention also includes any mutant or variant of the Btl109P or Btl260 strain which produces crystals, crystal proteins, protoxin or toxin having substantially the same properties, particularly insecticidal properties, as the Btl109P or Btl260

crystals, crystal proteins, protoxin or toxin. In this regard, variants of the Btl109P and Btl260 strains include variants whose total protein pattern is substantially the same as the protein pattern of either the Btl109P strain or the Btl260 strain as shown in FIG. 1.

This invention also is not limited to potato plants transformed with the truncated btl109P or btl260 gene. It includes any monocotyledonous or dicotyledonous plant, such as tomato, tobacco, rapeseed, alfalfa, sunflowers, cotton, corn, soybeans, brassicas, sugar beets and other vegetables, transformed with an insecticidally effective btl109P or btl260 gene part.

Nor is this invention limited to the use of Agrobacterium tumefaciens Ti-plasmids for transforming plant cells with an insecticidally effective btl109P or btl260 gene part. Other known techniques for plant cell transformations, such as by means of liposomes, by electropotation or by vector systems based on plant viruses or pollen, can be used for transforming monocotyledons and dicotyledons with such a gene part. For example, an insecticidally effective btl109P or btl260 gene part can be used to transform certain selected lines of corn and rice plants by methods such as are described by Fromm et al (1990), Gordon-Kamm et al (1990), Shimamoto et al (1989) and Datta et al (1990).

Furthermore, DNA sequences other than those present naturally in the Btl109P and Btl260 strains and encoding respectively the natural Btl109P and Btl260 protoxins, toxins and insecticidally effective protoxin portions can be used for transforming plants and bacteria. In this regard, the natural DNA sequence of these genes can be modified by: 1) replacing some codons with others that code either for the same amino acids or for other, preferably equivalent, amino acids; and/or 2) deleting or adding some codons; provided that such modifications do not substantially alter the properties, particularly the insecticidal properties, of the encoded Btl109P or Btll260 protoxin, the insecticidally effective portion of the Btl109P or Btl260 protoxin or the Btl109P or Btl260 toxin.

Also, other DNA recombinants containing the aforementioned DNA sequences in association with other foreign DNA, particularly the DNA of vectors suitable for transforming plants and microorganisms other than E. coli, are encompassed by this invention. In this regard, this invention is not limited to the specific plasmids containing the btl109P and btl260 genes, or parts thereof, that were heretofore described, but rather, this invention encompasses any DNA recombinants containing a DNA sequence that is their equivalent. Further, the invention relates to all DNA recombinants that include all or part of either the btl109P gene or the btl260 gene and that are suitable for transforming microorganisms (e.g., plant-associated bacteria such as Bacillus subtilis, Pseudomonas, and Xanthomonas or yeasts such as Streptomyces cerevisiae) under conditions which enable all or part of the gene to be expressed and to be recoverable from said microorganisms or to be transferred to a plant cell.

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SEQUENCE LISTING
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(iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:
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(A) LENGTH: 2411 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
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(B) STRAIN: BtI109P (DSM accession number 5870)
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: BtI260 (DSM accession number 5871)
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(B) LOCATION: 2..1045
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(B) LOCATION: 2..1045
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gene codes for a 66 kD insecticidal protein toxic
to Coleoptera."
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United States Patent *5,597,946*
Jaynes , et al. **January 28, 1997**

Method for introduction of disease and pest resistance into plants and novel genes incorporated into plants which code therefor

Abstract

A method of inhibiting pathogenic conditions of plants including viral, bacterial, and fungal infections and insert infestations by expressing into the plant genome genes encoding for a polypeptide inhibitor or inhibitor precursor of the pathogenic condition which inhibitor or precursor is selected from complementary oligonucleotides for blocking viral transcription or translation produced in vivo, one or more proteins derived from the humoral response to bacterial infection of the Hyalophora, an antifungal plasmid or a chitin integument disruption chitinase enzyme. Novel microbes, polypetides, and compositions containing amino acid sequences are disclosed.

Inventors: **Jaynes; Jesse M.** (Baton Rouge, LA); **Derrick; Kenneth S.** (Baton Rouge, LA)
Assignee: **Board of Supervisors of Louisiana State University and Agricultural and** (Baton Rouge, LA)
Appl. No.: **444762**
Filed: **May 19, 1995**

U.S. Class: **800/205**; 435/69.1; 435/70.1; 435/172.3; 435/200; 435/252.2; 435/320.1; 536/23.5
Intern'l Class: A01H 005/00; C12N 015/56; C12N 015/82; C12N 015/84; C12N 015/12
Field of Search: 435/69.1,70.1,172.3,200,252.2,320.1 800/205 536/23.5

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Parent Case Text

This application is a continuation of application Ser. No. 08/152,939, filed Nov. 15, 1993, now abandoned; which is a continuation of application Ser. No. 07/993,448, filed Dec. 16, 1992, now abandoned ; which is a continuation of application Ser. No. 07/845,348, filed Mar. 4, 1992, now abandoned; which is a continuation of application Ser. No. 07/373,623, filed Jun. 29, 1989, now abandoned; which is a continuation of application Ser. No. 06/889,225, filed Jul. 25, 1986, now abandoned;the entire disclosure of each of which are incorporated by reference.

Claims

1. A method of inhibiting a pathogenic fungal or bacterial plant condition, said method comprising: incorporating into a dicotyledonous plant genome one or more genes which encode for a polypeptide inhibitor or inhibitor precursor of said pathogenic condition, which inhibitor or inhibitor precursor is selected from the group consisting of attacins and cecropins.

2. The method of claim 1 wherein said incorporating is by infecting said plant with an Agrobacterium microbe containing said gene or genes.

3. The method of claim 2 wherein said Agrobacterium is Agrobacterium tumefaciens.

4. The method of claim 1 wherein said polypeptide is cecropin.

5. The method of claim 1 wherein said polypeptide is attacin.

6. The method of claim 1 further comprising incorporating into the genome of said plant a gene which encodes for lysozyme.

7. A plant produced according the method of claim 1 or claim 6.

Description

BACKGROUND OF THE INVENTION

This invention relates to a method for protecting plants from both disease and pests by means of genetic engineering to incorporate into the plant antagonistic agents or inhibitors for the infectious or harmful conditions which result. More specifically, plants suffer pathogenic conditions commonly known as diseases caused by virus, bacteria and fungus. Further, pests, such as various kinds of insects, cause untold damage to plants. The present invention provides a method for incorporating into the plant itself the means with which to deal with such pathogenic conditions.

Development of plant biology began in the early 1940s when experiments were being carried out to determine the biological principle causing formation of crown gall tumors. The tumor-inducing principle was shown to be a bacterial plasmid from the infective organism Agrobacterium tumefaciens. This plasmid has been characterized in exquisite biochemical detail utilizing the currently available techniques of recombinant DNA technology. The mode of operation for infection was the discovery that the bacterium elicits its response by actually inserting a small fragment of the bacterial plasmid into the plant nucleus where it becomes incorporated and functions as a plant gene. This discovery opened the door to using Agrobacterium and their plasmids as vehicles to carry foreign DNA to the plant nucleus. There are, however, limitations to the application of these techniques and they include: (1) susceptibility to infection with the Agrobacterium plasmid and (2) available tissue culture technology for regeneration of the transformed plants. These limitation have meant that, to date, there are no successful reports on genetic engineering of cereals because of the inability of

Agrobacterium to infect cereal plants.

The plant genes, like all other genes, are simply strings of nucleic acid bases. The function of synthetic genes within the plant can be to produce a gene product which has its own intrinsic value or to produce an intermediate gene product, such as mRNA, that plays a regulatory role within the plant cell. Synthetic genes are most useable when the technical capability does not exist to isolate and purify genes from natural organisms. Both purified and synthetic genes may be used in conferring protection to plants against disease or pests. An example of the use of synthetic genes to confer resistance to viruses and viroids in plants comes through the use of the coevolution of many viruses with the plant hosts. Plants and animals have evolved very precise and elegant mechanisms which allow the regulated expression of their genes. Viruses by co-evolution have exploited and continue to exploit the eukaryotic cell of a plant by mimicking the general structure of the plant genes. Often, the end result of this for the plant is disease. While plants and animals are prisoners of their own evolution, so are viruses since they are dependent upon the plant and animal system of gene regulation and expression to synthesize and translate their own genes into the plant. This dependency is considered the key to control of viral disease. It has recently been found that bacteria regulate expression of some genes in a rather novel way. Under conditions where the cell would repress the biosynthesis of a particular protein, an additional level of control is exerted. This newly discovered type of gene control is called "micRNA" control and stands for "mRNA-interfering complementary RNA". This micRNA or "antisense" RNA is complementary to the 5' end of the gene and when it is produced has the ultimate effect of reducing the amount of messenger RNA (mRNA) by annealing to it, thus removing it from normal protein synthesis.

Viroids are single-stranded ribonucleic acid (RNA) of a few hundred nucleotides. They are the smallest self-replicating structures known and represent the lowest form of life. They are the causative agents of a number of plant diseases and elicit mild to lethal responses in a range of plants depending on the fine structure of the viroid and the susceptibility of the plant genotype. In the case of viroids, the logic is to inhibit the replication of the disease-causing molecule. Viroids are infective pieces of nucleic acid and do not have a protein component like viruses. Using methodology similar to viruses, we have the opportunity of blocking the nucleic acid replication (called transcription).

Many plants contain genes that confer virus resistance and, in some cases, resistance is due to a single dominant gene while, in other cases, the resistance is genetically more complex, i.e., requiring a number of genes to confer resistance. While it is presently practically impossible to identify, isolate and purify these resistance genes and, in fact, the chromosomes which carry these genes cannot even be located, utilizing the in vivo-produced antisense fragments, it may be possible to attain virus and viroid resistance by the insertion of a single synthetic gene which ultimately would produce an RNA complementary to specific regions of the viral genome and would play a role in the disruption of replication or translation of the virus. This complementary or antisense gene would be specific for a particular viral pathogen. Plants endowed with a number of these antisense genes would protect them from a variety of viral diseases, such as the symptoms observed in a viral disease of a potato.

In contrast to the use of one or more synthetic genes for protection of plants against viral disease, bacterial protection employs the known response of an insect to bacterial infection to confer resistance to bacterial disease. The pupae of Hyalophora (a type of silk moth) respond to bacterial infection by the synthesis of mRNAs which culminate in the production of about 15 to 20 new proteins. Lysozyme, the antibacterial protein found in egg white and human tears, and two other classes of antibacterial peptides, called cecropins and attacins, have been purified from these newly synthesized proteins. Lysozyme has been shown to be effective in limiting bacterial growth. When lysozyme was placed on a small paper dish in two concentrations, after an agar plate was seeded with plant pathenogenic bacteria, the lysozyme inhibitory zone was quite clear.

These proteins have a rather broad spectrum activity in that they are effective on many different types of bacteria. Thus, the insects have evolved a rather successful and novel means to fight bacterial infections. Although a traditional immunologist would think this system lacks specificity, the insect has a rather potent arsenal of at least three different bacterial proteins which may work in different ways to actively seek to destroy the bacterial pathogen. Thus, the invading bacteria is presented with a formidable challenge which would be very difficult to circumvent. While a bacterial pathogen may be naturally resistant to one, it is highly improbable that it would be resistant to all three toxins. Although determination of the exact mode of action of the protein toxins is required, they are quite prokaryote specific and appear to be benign to eukaryotic cells. Incorporation of the proteins derived from humoral response An Hyalophora are an attractive genetic system for protecting plants from bacterial disease which causes significant economic loss. As an example, the main diseases in potato are bacterial soft rot and bacterial wilt caused by Erwiria carotovora and Pseudomona solanacearum, respectively. These diseases are primarily responsible for limiting the growth of potatoes in many areas of Asia, Africa, South and Central America. The introduction of genes encoding for these antibacterial proteins into crop plants may revolutionize the protection of plants from bacterial-produced disease.

In a manner analogous to the antibacterial-protein producing insect, it has been found that some bacteria are natural repositories which contain genes encoding for proteins effective against fungi. The biochemical analysis of these compounds and the ultimate molecular characterization of genes encoding the synthesis of these natural antifungal agents could be of great importance in limiting the scope and severity of fungal disease in crop plants. It is believed that from 1845 to 1860 the fungal disease of the potato caused by Phytophthora infetans or Late Blight caused the Irish potato famine in which one million people died of starvation and one and a half million people immigrated to North America because of the decimation of the potato crop caused by this fungal pest.

Control of insect pests which cause tremendous losses of food and fiber derived from plants directly attributed to the insects and as a vector for the infection and spread of other plant pathogens requires increasing a plants tolerance to insect damage. Such increased tolerance would be of significant economic value. One method for protecting plants from insect damage is the use of natural insecticides such as a protein isolated from Bacillus thuringiensis which forms a high molecular weight crystal in the bacteria which is toxic to the larvae of a number of

lepidopteral insects. However, it is believed that the insects would develop an immunity to the toxin within a few generations. Thus, other possibilities are being considered. One of the most promising is the use of an enzyme called chitinase as a natural insecticide. Chitinase is an enzyme produced in bacteria and the gene which encodes it has been isolated. An insect exoskeleton and gut are composed of chitin and it has been shown that any disruption of the chitin integument will result in the insect contracting an infection from the natural environment with death ensuing after a very short period of time. Inserting the enzyme-encoding gene for chitinase into the plant will provide a potent chitin-dissolving enzyme within the plant which will limit the extent of damage wrought by insects and secondarily limit the spread of other plant diseases which use insects as a vector.

SUMMARY OF THE INVENTION

In a broad aspect this invention provides a method of inhibiting a pathogenic plant condition which method comprises expressing into the plant genome an inhibitor for said condition derived by ligating from a natural or synthesized DNA source, a gene encoding for one or more polypeptide inhibitors or inhibitor precursors for said condition, inserting said gene into a plant vector and inserting said plant vector into a plant to be protected from the pathogenic plant condition.

More specifically, the present invention provides a method of inhibiting a pathogenic plant condition selected from viral infection, bacterial infection, fungal infection or insect infestation in a plant, said method comprising expressing into the plant genome one or more genes which encode for a polypeptide inhibitor or inhibitor precursor of said pathogenic condition, which inhibitor or precursor is selected from (a) a complementary oligonucleotide for blocking viral transcription or translation produced in vivo, (b) one or more proteins derived from the humoral response to bacterial infection of the Hyalophora, (c) an antifungal plasmid, and (d) a chitin integument disruption chitinase enzyme whereby such disruption results in subsequent infection from the environment of an insect feeding on said plant, said expressing being carried out by random ligation of a plasmid containing said inhibitor or precursor encoding gene, uptake of the inhibitor containing plasmid into Escherchia coli having a Hind III 17 fragment of T-DNA incorporated therein, introducing into an Agrobacterium strain carrying an unmodified R1 plasmid such that culturing with the plant genome results in said T-DNA containing the passenger (synthetic) DNA being incorporated into the plant genome and can be regenerated therewith to have an inhibiting effect for the pathogenic condition.

An alternative method of expressing the gene encoding for one or more polypeptide inhibitors or inhibitor precursors includes the ligation of such gene and insertion thereof into a plant vector identified as pMON237.

A more specific method of inhibiting or preventing virus production in plants ordinarily susceptible to said virus, which method comprises the expression into the plant gene of a complementary oligonucleotide for blocking the translation of said virus.

An additional aspect of the invention is a method of inhibiting or preventing bacterial disease in

plants which includes expression of one or more genes encoding for one or more proteins derived from the humoral response to bacterial infection of the Hyalophora into the genome of a plant susceptible to bacterial infection.

A still further feature of the present invention includes a method of inhibiting fungal infections of plants ordinarily susceptible thereto, which method comprises expression of genes encoding for bacterially-derived proteins effective to inhibit growth of fungi.

A still further aspect of this invention provides a method for inhibiting damage to economically important crop or fiber plants from larvae of lepidopterans, which method comprises expressing the genes which code for chitinase enzyme into said crop or fiber plants such that upon ingestion by said larvae the chitinase enzyme disrupts the chitin integument of the larvae whereby such disruption results in subsequent infection, from external environmental sources.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The method provided by the present invention provides an inhibitor for a number of adverse plant conditions, including bacterial infections, viral infections, fungal infections, and insect infestations. Initially, the method for inhibiting plant disease, particularly plant disease caused by bacterial infections, is considered. Bacterial infections are known to cause an antibacterial response in certain insects. As indicated above, the pupae of Hyalophora synthesize mRNA's which result in the production of 15 to 20 new proteins, from which lysozyme, cecropin and attacin have been purified.

The lysozyme gene and protein was obtained from Hyalophora-derived plasmid pBR322, provided by Kleanthis Xanthopoulos. The lysozyme gene was removed from the plasmid pBR322 by digestion with the enzyme Pst I. The resultant fragment was purified and treated with the Bal 31 enzyme to remove the 3' poly dG tail. Then the adapter shown as follows:

```
GTTTCATGAAACAGATCTGTCGACAGATCTGTTTCATGAAAC
CAAAGTACTTTGTCTAGACAGCTGTCTAGACAAAGTACTTTG
```

was ligated to the fragment after digestion with enzyme Xmn I. Then the fragment is digested with Sal I and cloned into the plasmid pBR322. The lysozyme gene was rescued by digestion with enzyme Bgl II and inserted into the plant vector pMON237.

The lysozyme gene coding for antibacterial proteins has been identified and contains the following nucleotide sequence:

```
AGATCTGTTTCATG AAA CGTTTCACG AGA TGCGGG
TTA GTG CAG GAG CTTAGG AGA CGA GGCTTCGATGAA ACTTTG ATG AGTAACTGG
GTCTGCCTTGTCGAG AACGAA AGCGGA CGG TTTACCGATAAA ATCGGTAAA GTT
AACAAG AACGGA TCTCGA GACTACGGCCTCTTCCAG ATCAATGACAAA TACTGG
```

```
TGCAGTAAG GGA TCCACTCCTGGA AAG GATTGCAACGTG ACTTGTAATCAG CTA
CTG ACTGACGACATTAGCGTG GCA GCTACG TGCGCG AAG AAG ATTTACAAA CGC
CACAAG TTTGACGCTTGG TACGGA TGG AAA AATCACTGTCAA CATGGA CTG CCA
GATATTAGCGACTGTTAG AGACGACTTATTATAGCCTTCGTTTCATGAAAC
AGATCT.
```

This lysozyme gone produces an accompanying protein or polypeptide which has the amino acid sequence:

```
Lys Arg Phe Thr Arg Cys Gly
Leu Val Gln Glu Leu Arg Arg Arg Gly Phe Asp Glu Thr Leu Met Ser Asn Trp
Val Cys Leu Val Glu Asn Glu Ser Gly Arg Phe Thr Asp Lys Ile Gly Lys Val
Asn Lys Asn Gly Ser Arg Asp Tyr Gly Leu Phe Gln Ile Asn Asp Lys Tyr Trp
Cys Ser Lys Gly Ser Thr Pro Gly Lys Asp Cys Asn Val Thr Cys Asn Gln Leu
Leu Thr Asp Asp Ile Ser Val Ala Ala Thr Cys Ala Lys Lys Ile Tyr Lys Arg
His Lys Phe Asp Ala Trp Tyr Gly Trp Lys Asn His Cys Gin His Gly Leu Pro
Asp Ile Ser Asp Cys.
```

In a somewhat similar manner, the attacin gene and its accompanying protein, obtained as a part of the plasmid pBR322 from Kleanthis Xanthopoulos, were removed, provided with appropriate start and stop amino acids and inserted into a plant vector for inclusion into a suitable host for growth and testing for antibacterial properties in plant cells. The attacin gene was removed from pBR322 by digestion with the enzyme Pst I, according to conventional procedures. The resultant plasmid fragment was purified and digested with FnuDII and Dra I. The adapter oligonucleotide

```
CGTCCATGGACGAGATCTGTCGACAGATCTCGTCCATGGACG
GCAGGTACCTGCTCTAGACAGCTGTCTAGAGCAGGTACCTGC
```

was joined to the fragment by ligation. Then the adapter-containing fragment was digested with Sal I and cloned into plasmid pBR322. The full length attacin gene was then obtained from pBR322 by digestion with Bgl II and inserted into the plant vector pMON237 and had the start methionine at the amino terminus end. The plant vector pMON237 containing the antibacterial attacin gene confers this antibacterial property on plants produced from plant cells having the pMON237 inserted.

The attacin gene coding for antibacterial protein has been identified and contains the following nucleotide sequence:

```
AGATCTCGTCCATG
```

```
GACGCG CACGGA GCCCTTACG CTCAACTCCGATGGTACCTCTGGTGCTGTG GTT
AAA GTA CCCTTTCGTGGTAACGACAAG AATATA GTA AGCGCTATCGGTTCCGTA
GACTTA ACTGATAGG CAG AAA CTA GGCGCTGCA ACCGCTGGA GTG GCA CTG GAT
AATATA AACGGTCACGGA CTA AGTCTCACG GATACA CACATCCCCGGG TTCGGA
GACAAG ATG ACA GCA GCCGGCAAA GTG AATGTCTTCCACAATGATAACCACGAC
ATCACA GCG AAG GCTTTCGCCACCAGA AACATG CCG GATATTGCTAATGTA CCT
AATTTCAACACTGTCGGTGGCGGA ATA GACTATATG TTCAAA GATAAG ATTGGT
GCA TCTGCG AGCGCCGCTCACACG GACTTTATCAATCGCAACGACTACTCTCTT
GACGGG AAA CTG AACCTCTTCAAG ACTCCTGATACCTCG ATTGATTTCAACGCC
GGTTTCAAG AAG TTCGATACA CCTTTCATG AAG TCCTCTTGG GAG CCTAACTTC
GGA TTCTCA CTTTCTAAA TATTTCTGA TTA GTATTTTAATTTTAATTCTATATATATAAA
TTTAGATGTATATGTATATATATATATTTTTTTTTTATTAATATGATATCACTAAATGTATTTACTCCTTC
GATTATTATTACTTTTTTTGTTTCGTCCATGGACGAGATCT.
```

This attacin gene produces an accompanying protein or polypeptide having the amino acid sequence as follows:

```
Asp Ala His Gly Ala Leu Thr Leu Asn Ser Asp Gly Thr Ser Gly Ala Val Val
Lys Val Pro Phe Ala Gly Asn Asp Lys Asn Ile Val Ser Ala Ile Gly Ser Val
Asp Leu Thr Asp Arg Gln Lys Leu Gly Ala Ala Thr Ala Gly Val Ala Leu Asp
Asn Ile Asn Gly His Gly Leu Ser Leu Thr Asp Thr His Ile Pro Gly Phe Gly
Asp Lys Met Thr Ala Ala Gly Lys Val Asn Val Phe His Asn Asp Asn His Asp
Ile Thr Ala Lys Ala Phe Ala Thr Arg Asn Met Pro Asp Ile Ala Asn Val Pro
Asn Phe Asn Thr Val Gly Gly Gly Ile Asp Tyr Met Phe Lys Asp Lys Ile Gly
Ala Ser Ala Ser Ala Ala His Thr Asp Phe Ile Asn Arg Asn Asp Tyr Ser Leu
Asp Gly Lys Leu Asn Leu Phe Lys Thr Pro Asp Thr Ser Ile Asp Phe Asn Ala
Gly Phe Lys Lys Phe Asp Thr Pro Phe Met Lys Ser Ser Trp Glu Pro Asn Phe
Phe Ser Leu Ser Lys Tyr Phe.
```

Further, and similarly, the antibacterial protein producing cecropin gene, obtained in plasmid pBR322 received from Kleanthis Xanthopoulos was first cut with restriction enzymes Pst I and HinPII to provide a plasmid fragment pCPFL1. The resulting 260 base pair fragment was purified and treated with T4 DNA polymerase to fill in the HinPII site. The resultant fragment was then treated with T4 DNA ligase and the synthetic adapter C3, which is identified as follows:

```
CTAGCATAAAGATCTGACGTCAGATCTTTATCCTAG
GATCGTATTTCTAGACTGCAGTCTAGAAATAGGATC,
```

was joined to the fragment. The new gene fragment was then ligated to pBR322 which had been cleaved with restriction enzymes XmnI and AatII. Clones containing the correct ampicillin sensitive genotype were selected, cut with XmnI and ligated with a synthetic adaptor identified as C5, which has the following nucleotide sequence:

```
CTTTCCATTTCATGGTAGATCTACCATGAAATGGAAAG
GAAAGGTAAAGTACCATCTAGATGGTACTTTACCTTTC
```

The resultant fragment was retransformed with E. Coli. The cecropin gene is rescued from E. Coli by digestion with Bgl II and inserted into the plant vector pMON237. Thus, the cecropin gene is regenerated without its leader peptide and with an appropriate start methionine at the amino terminus end and the correct translational termination (stop) signal at the carboxy terminus end.

The cecropin gene has been identified and has the following nucleotide sequence:

```
AGATCTACCATGAAATGGAAAGTCTTCAAGAAA
ATTGAA AAA ATG GGTCGCAACATTCGA AACCGTATTGTCAAG GCTGGA
CCA GCG ATCGCG GTTTTA GGCGAA GCCAAA GCG CTA GGA TAA AGATCT.
```

This cecropin gene produces an accompanying protein or polypeptide having an antibacterial amino acid sequence as follows:

```
Lys Trp Lys Val Phe Lys Lys
Ile Glu Lys Met Gly Arg Asn Ile Arg Asn Gly Ile Val Lys Ala Gly
Pro Ala Ile Ala Val Leu Gly Glu Ala Lys Ala Leu Gly.
```

The Agrobacterium tumefaciens microbes containing the insect produced antibacterial proteins produced according to the method of the present invention have been given the designations pAT-LYS, pAT-ATN and pAT-CN for those which contain genes encoding for lysozyme, attacin and cecropin proteins, respectively. These novel microbes are available for reproduction and maintenance and will be preserved by the inventor at Louisiana State University, Baton Rouge, Louisiana, until such time as deposit in a commercial depository is required in the event of allowance of the present application. In view of the present discoveries and inventions, another feature of this invention is a composition comprising a plasmid contained in a microbe which contains a DNA sequence which codes for a polypeptide derived from the humoral response to bacterial infection of the Hyalophora. Particularly, the present invention includes a composition in which the microbe is Agrobaeterium tumefaciens. More particularly, the composition of this invention includes such a microbe in which the polypeptide is selected from lysozyme, attacin, cecropin and a mixture thereof.

The method of inhibiting fungi includes the selection, cloning and insertion of genes encoding for antifungal compounds into an appropriate plant vector. Certain naturally occurring bacteria

produce toxins for fungi. Such bacteria retain gene(s) which encode for the production of these antifungal compounds. The DNA separated from these antifungal compound producing bacteria are isolated, shotgun cloned into a lambda vector and subsequently used to transfect E. Coli. In addition, the same DNA can be shotgun cloned into Pseudomonas directly using the Pseudomonas vectors pWS3 and pWS6 described by Wyerneke et al, Gene 38 (1985) 73-84. The resultant transformants are plated and oversprayed with the indicator single cell eukaryote Rhodotorula. This powerful selection tool locates the DNA (genes) encoding for the antifungal toxin compounds and characterizes them sufficiently to allow for their expression in a plant by suitable plant vectors in the manner previously described. Insertion of the antifungal compounds as genes or DNA encoding therefor provides plant species having antifungal properties.

In much the same manner a species providing a chitinase enzyme is selected for identification, cloning, insertion into a plant vector and production of a plant producing the chitinase enzyme. It has been found that certain species of the bacterial genus Vibrio produce a very active chitinase enzyme. Thus another aspect of this invention provides a method for inhibiting insect infestation and insect damage to plants by providing a chitinase enzyme producing plant. The DNA or gene encoding for production of the chitinase enzyme can be cut out of the Vibrio bacteria DNA with digestion by the restriction enzyme Hind III. A DNA sequence analysis can be employed to determine the appropriate start and stop positions of the gene. Further cloning is required to implant the desired gene into the plant genome as described previously for the antibacterial encoding genes inserted into plant vectors.

The method for inhibiting viruses and viroids includes the provision of an antisense or complementary oligonucleotide which inhibits or prevents the replication of the virus or viroid or which inhibits the translation of the virus. In Vitro procedures utilizing a 41 base DNA oligonucleotide having the sequence:

GATCTCCACGGTTGTGGCCATATAATCATCGTGTTTTTCAA

effectively blocked 98% of the translation of a virus genome. This procedure was carried out by hybridizing the DNA to the virus in an 8 microliter reaction mixture containing 20 mM Hepes, pH 7.6, 0.1M NaCl and 1 mM ETDA. RNA concentration of the virus was 0.5 mg/ml and the DNA was added in a five-fold molar excess. In general, the reaction mixtures were heated at 70.degree. C. for 10 minutes followed by incubation at 45.degree. C. for 3 hours. The process of determining viral RNA translation is in a cell-free protein synthesis regime, such as in RNA rabbit reticulocyte lysate system described by Shih et al at Proceedings of the National Academy of Science of the U.S.A., 75, 5807-5811 (1978) and in the Journal of Virology, 30, 472-480 (1979), both of which are incorporated by reference as if fully set forth. As a result of the hybridization, viral translation was effectively blocked.

In the case of viroids, replication was prevented in the potato spindle tuber viroid (PSTV) by

hybridization of synthetic DNA fragments to the PSTV in the central conserved region which appears to be present in all known viroids and is presumed to be important for replication. The synthetic DNA fragments have the oligonucleotide sequence and identification as follows:

```
GATCTAGGGATCCCCGGGGAAACCTPSTV1
GATCTAGGTTTCCCCGGGGATCCCTPSTV2
```

This hybridization was carried out by annealing the various oligonucleotide fractions to purified, infectious PSTV RNA. The sample mixture was heated to 90.degree. C. for 5 minutes and then allowed to cool slowly to room temperature. These mixtures were then innoculated onto PSTV sensitive tomato plants and symptoms were allowed to develop. The results are shown in the Table below.

Table of Molar Ratio of Compositions Innoculated in Tomato Plants

Innocula	Molar Ratio	Infected Tomato Plants (#infected/#innoculated)
PSTV + PSTV1	1:1	0/4
PSTV + PSTV1	10:1	0/4
PSTV + PSTV1	1:10	0/4
PSTV + PSTV2	1:1	0/4
PSTV + PSTV2	10:1	2/4
PSTV + PSTV2	1:10	0/4
PSTV + PSTVf*	1:1	1/4
PSTV + PSTVf	10:1	0/4
PSTV + PSTVf	1:10	0/4
PSTV alone		3/5

*PSTVf is a full length DNA of PSTV.

When hybridization occurs, the further replication of the PSTV molecule was blocked.

The synthetic DNA transcription blocking for viroids and synthetic DNA translation blocking for viruses are inserted into a plant vector to produce plants which are not susceptible to the viroids and viruses described.

Having described the invention, one skilled in the art will be aware of variations and changes

therein which are within the scope and spirit of the present invention. Therefore, it is desired that the invention be limited only by the lawful scope of the following claims.

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